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Radial ring width and wood structure in the ozone-exposed Norway spruce seedlings grown under different nitrogen regimes

Sari Makkonen*, Kirsi Huuhilo, Jarkko Utriainen, Toini Holopainen and Pirjo Kainulainen

*Department of Environmental Science, University of Eastern Finland, P.O. Box 1627, FI-70211 Kuopio, Finland (*corresponding author's e-mail: sari.makkonen@uef.fi)*

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Norway spruce seedlings were exposed to two ozone levels (ambient and 1.5–1.6 × ambient) at moderate (optimum N), low (70% of optimum N), and high soil-nitrogen availability (150% of optimum N), in an open-air exposure field for two growing seasons. Radial ring width and wood structure in the stem were investigated from the seedlings. The seedlings grown under elevated O_3 showed an increase in radial width of growth ring built during the first exposure season. Elevated O_3 increased latewood width, its proportion within the growth rings being most pronounced in low N treated trees, whereas higher N mitigated latewood production. High soil N increased earlywood width and resulted in an increased lumen area and lower cell-wall/cell-lumen ratio of tracheids within earlywood and latewood. Ozone had no effect on earlywood width or the lumen area or cell wall/ cell lumen ratio in juvenile wood. High latewood percentage in O_3-exposed seedlings implies earlier transition from earlywood to latewood or longer period of latewood formation.

Introduction

Target values of tropospheric ozone (O_3) for protection vegetation and forest were frequently exceeded in many European regions during the last decades (Langner *et al*. 2012a), and surface O_3 concentrations in the northern hemisphere are projected to increase until 2100 due to future climate warming (Royal Society 2008, Langner *et al*. 2012b, Klingberg *et al*. 2014). The European boreal region is affected by long-range transport of O_3 and its precursors from the main source regions in continental Europe (Langner *et al*. 2012a). Changes in atmospheric circulation due to climate change can therefore affect future levels and O_3 deposition (Langner *et al*. 2012a) into boreal forest.

Several studies have documented the harmful effects of ozone (O_3) on photosynthesis and the growth of boreal-forest tree species (Karlsson *et al*. 2002, Karlsson *et al*. 2007, Wittig *et al*. 2009, Huttunen and Manninen 2013). Fast-growing deciduous trees have been found to respond earlier to O_3 exposure than slow-growing coniferous species (Fuhrer *et al*. 1997). This has been related to differences in water uptake and stomatal conductance that mainly affect and regulate the O_3 uptake of leaves (Skärby *et al*.

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1998). Also the plant's capability to detoxify oxygen radicals determines the appearance of O_3 injury (Kronfuß *et al*. 1998). Visible symptoms in conifer needles (Kivimäenpää *et al*. 2014) as well as a decline in growth and photosynthesis occurred after more than one year exposure to O_3 (Lucas and Diggle 1997, Langebartels *et al*. 1998, Wallin *et al*. 2002). The findings suggest that the harmful effects of O_3 mainly result from the cumulative exposure over a long time (Lucas and Diggle 1997, Langebartels *et al*. 1998, Wallin *et al*. 2002).

The importance of the interaction of O_3 with other simultaneously-occurring environmental stressors including the intensity of each stress factor has been increasingly emphasised in the exposure studies (Skärby *et al*. 1998, Manes *et al*. 2001, De Vries *et al*. 2014). Trees grown under optimal conditions have been found to be more sensitive to O_3 than those grown under stress (Lippert *et al*. 1996, Fuhrer *et al*. 1997). Drought stress and the deficiency of nitrate, phosphate, and sulphate, may cause lower stomatal conductance (Clarkson *et al*. 2000) and lead to reduced O_3 uptake by plant. However, increased susceptibility to O_3 of tree seedlings grown under N deficiency has also been reported (Pääkkönen and Holopainen 1995, Utriainen and Holopainen 2001a).

It is well-established that changes in the tree growth rate affect the formation and anatomical characteristics of wood (Zobel and van Buijtenen 1989). Young trees are especially responsive to environmental and growth factors that influence juvenile wood properties (Zobel and van Buijtenen 1989). Ozone is likely to affect the stem growth indirectly through reduction in resources, particularly carbohydrate availability for growth (Weber and Grulke 1995). In hardwood species, O_3 has been observed to have a negative effect on the wood formation. Ozone exposure over one growing season reduced the size of phloem ray cells in the stem of low-fertilized *Betula pendula*, and also inhibited the growth of xylem tracheids and ray cells, regardless of fertilization (Matyssek *et al*. 2002). Three-year exposure to an elevated O_3 concentration reduced the distance from pith to bark and the lumen diameter of vessels and fibers, as well as increased wall thickness and wall percentage in the current year's annual ring in the stem of *Populus tremuloides* clones (Kaakinen *et al*. 2004). Also in clonal *B. pendula*, O_3 increased cell wall percentage and decreased vessel percentage (Kostiainen *et al*. 2006). Eleven-year follow-up data of the hardwood trees (Kostiainen *et al*. 2014) revealed aspen to be more sensitive to elevated O_3 than birch, exhibiting decreased radial growth and cell diameters, increased vessel density and proportion in particular within early juvenile wood.

Few studies focused on the wood formation and wood cell properties of juvenile wood in Norway spruce seedlings (Kurczyńska *et al*. 1998) or mature wood in adult spruce grown under O_3 stress (Karlsson *et al*. 2006, Wipfler *et al*. 2009). In the Norway spruce seedlings grown on nitrogen-enriched soil, the exposures to elevated O_3 concentrations resulted in an increase in tracheid diameter and wall thickness, and reduction in tracheid number in the latewood (Kurczyńska *et al*. 1998). Studies on adult Norway spruce revealed O_3-induced decline in the stem diameter growth (Karlsson *et al*. 2006, Wipfler *et al*. 2009) but not necessarily in the volume growth of spruce which was even found to slightly increase due to the increase in height growth (Pretzsch *et al*. 2010).

The research described here is part of a study addressing the impacts of slightly-elevated O_3 and nutrient imbalance on height growth and needle characteristics including chloroplast pigments, stomatal conductance, and ultrastucture of the mesophyll cells, in four-year-old Norway spruce seedlings (Utriainen and Holopainen 2001b). Treatments were applied in an open-field ozone fumigation facility during two growing season in 1997 and 1998. The aim of the present study was to determine the effect of O_3 and N availability on the radial stem width, wood formation, and the structure of tracheid cells in the stem of the experimental seedlings. Both O_3 and nutrient supply are capable of affecting photosynthates such as starch and sucrose of needles (Utriainen and Holopainen 2001b, Riikonen *et al*. 2012, Kivimäenpää *et al*. 2014) and carbon allocations in trees (Huttunen and Manninen 2013). Thus, we hypothesized that O_3 and soil nitrogen can alter the cambial activity and wood formation in the stem of spruce seedlings, which

is reflected in ring width, earlywood and latewood ratio, and the structure of tracheid cells within growth rings build during two years of exposure.

Material and methods

Experimental design

Ozone exposure and N fertilization experiment was conducted in an open-air exposure field at the Research Garden of the University of Kuopio (62°54´N, 27°40´E) during the summers of 1997 (30 May to 9 Sep.) and 1998 (18 May to 28 Sep.). Altogether 180 four-year-old Norway spruce (*Picea abies*) seedlings (T9-92-06) originating from central-eastern Finland, were planted in plastic pots (7.5 l) containing mixture (3:1 v/v) of quartz sand and fertilized *Sphagnum* peat (Vapo peat PP6, 1 kg m^{-3}: N-P-K (12-9-18) with micronutrients in late May 1997 (Utriainen and Holopainen 2001b).

The seedlings were randomly divided among two control (ambient air) and two ozone (elevated O_3) plots (diameter approx. 10 m). Each plot consisted of 45 seedlings (3 nutrition treatment × 15 seedlings per treatment). Ozone was produced from pure oxygen with an O_3 generator (Fischer OZ 500, Fischer, Bonn, Germany), and released to the exposure area through perforated plastic tubes in the upwind direction. The ozone concentration was monitored with a model 1008-RS O_3 analyzer (Dasibi Environmental Corp., Glendale, California). Mean 24-hour O_3 concentrations within the O_3 plots were approximately 1.6 × the ambient concentration during the growing season in 1997 and 1.5 × ambient in 1998. Ozone concentrations, and an exposure index of AOT40 [Accumulated O_3 exposure over the threshold of 40 ppb or nl l^{-1} (80 μg m^{-3})] for daylight hours (> 50 W m^{-2}), and 24 h day^{-1} are given in Table 1 and described more detailed by Manninen *et al*. (2002).

Fertilizing was started one week after planting. The seedlings were treated three times a week with three N concentrations (15 seedlings per treatment): low N (25 mg N l^{-1}) (LN), moderate N (50 mg N l^{-1}) (MN), and high N (100 mg N l^{-1}) (HN). Additional irrigation was given if needed. The treatment solutions were prepared in nutrient solution designed for the culture of conifers (Ingestad 1962, Palomäki and Holopainen 1994). The detailed nutrient composition of treatments is described elsewhere (Utriainen and Holopainen 2001b). In addition to the nutrient solution, seedlings were exposed to ambient rain. For overwintering in the exposure field the pots were sheltered with spruce branches and snow.

Needle nitrogen concentrations

Needles of all fully-grown current- (1998) and previous-year (1997) flushes were taken from five experimental seedlings in each N treatment per ambient and O_3 plots in early October 1998 (Utriainen and Holopainen 2001b). Samples were dried at 70 °C for 48 h, weighed, grounded and wet digested. Needle N concentration was analysed using a standard Kjeldahl method (Allen 1989). Samples (100 g) were digested using concentrated H_2SO_4 and selenium Kjeltabs (3.5 g K_2SO_4 + 35 mg Se) catalyst mixture. Ammonia was liberated by distillation of a

Table 1. Summary of the mean AOT40 values measured over daylight hours (> 50 W m^{-2}) and the full 24 h day^{-1}, and the mean 24-h O_3 concentrations (ppb) in ambient and elevated O_3 plots during the growing seasons (May–Sep.) in 1997 and 1998.

	1997		1998	
Treatment	Ambient	Elevated O_3	Ambient	Elevated O_3
AOT40 (ppm h)				
Daylight	0.5	6.9	0.1	2.8
24 h	0.8	11.7	0.3	10.2
24 h O_3 concentration (ppb)	22.2	35.6	22.4	33.4

digest with 32% NaOH (30 ml) and absorption in 2% H_3BO_3 (20 ml). The ammonium borate formed was titrated back to H_3BO_3 with standard 0.01 M HCl.

Wood analysis

Two stem samples were collected from seven experimental seedlings in each N treatment per ambient and O_3 plots. Approximately 1-cm-thick segments were cut from the fifth annual growth of the main stem. The stem segments were immediately frozen and stored at –20 °C until preparing for microscopical studies. All samples were prepared and analysed during 1998 and 1999. Samples contained the previous four growth rings and the fifth developing annual layer with the cambial zone and adjacent phloem. To estimate the radial growth variations of seedlings, stem's radius from pith to the stem surface, and the radial width of growth rings were analysed. The changes in the wood structure were determined by measuring tracheid length, lumen area and cell wall/cell lumen ratio within earlywood and latewood, and the earlywood and latewood percentages and widths in growth ring.

For the analysis of the stem's radial width and wood-cell properties in the 1997 and 1998 growth rings, the entire stem disk was softened in boiling water for an hour. The wet sample was frozen and 16-μm-thick transverse sections were cut with a cryo-microtome. The sections were mounted on slides and stained with a mixture of safranin and alcian blue (1:2). For tracheid length measurements, the 1997 and 1998 growth rings were separated with a scalpel under a stereomicroscope and macerated in a solution of acetic acid and hydrogen peroxide (1:1) for one day. Earlywood is characterized by short tracheids with large inner diameter and thin wall, while latewood is composed of long narrow diameter tracheids with thick cell walls. The wood sections and tracheid lengths were investigated with a Zeiss-Axiolab light microscope (Carl Zeiss, Jena, Germany) and a PC computer equipped with the image analysis software (Scion Image for Windows). Digital pictures were taken with a CCD colour video camera (COHU RGB, 2252-1040). For the analysis of cell wall/cell lumen ratio and average cell lumen area, three randomly selected pictures (139 × 103 μm) were taken from both earlywood and latewood bands on the stem sections.

The stem radius and the width of growth rings formed during 1997 and 1998 growing seasons were measured and the percentage of latewood was calculated as a ratio of latewood area to the total growth ring area. Each ring area (RA) was obtained from a circular ring area equation

$$RA = \pi(R^2 - r^2) \quad (1)$$

where R is the radius from pith to outer edge of the growth ring and r is the radius from pith to the inner growth-ring boundary. The widths of growth rings and the entire stem were measured along the radius from pith to each ring's edge and the stem surface, respectively. The values used in the analysis were based on three replicate measurements carried out with an ocular measuring scale made at an approximately equal angle of 120° from each stem cross-section.

The width of phloem from the cambial zone to the outer surface of the phloem layer, the width of the cambial zone between phloem and xylem, and the number of fusiform cells within the cambial zone were measured from five experimental seedlings from each N treatment per ambient and O_3 plots. Five tissue samples from fifth annual growth of the main stem containing phloem, cambial zone and outer xylem, were cut with a scalpel from the stem segment and pre-fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) and postfixed in 1% buffered OsO_4 solution. Samples were dehydrated in ethanol, embedded in Ladd's LX-112 resin. The semithin cross-sections (thickness 1.0 μm) were cut with an Ultracut E (Reichert-Jung AG, Wien, Austria) and stained with toluidine blue. The mean width of phloem (n = 15) was based on three replicate measurements carried out with an ocular measuring scale on five tissue sample using. The mean width of cambial zone was measured and the number of fusiform cambial cells counted along ten radial cell rows from three pictures (203 × 143 μm) in each tissue sample using image analysis (Scion Image for Windows). In total, 150 radial files per seedling were analyzed. Radially flattened and thin-walled cambial

cells were easily distinguished from thick-walled latewood tracheids and differentiated secondary phloem.

Statistical analyses

Each individual seedling representing different fertilization levels had totally randomized location inside the control and O_3 plots. The factorial design of the experiment consisted of two levels of O_3 (ambient and elevated) and three levels of nitrogen (LN, MN and HN) in each plot. The data were analysed with a GLM procedure of SPSS (IBM SPSS Statistics). The results from two plots under the same ozone treatment were combined.

The relationship between the needle nitrogen concentration and the characteristics of the stem structure determined from the same seedlings was analysed using Pearson's correlation except for the percentage of latewood which was analyzed using Spearman's correlation. Correlations of radial width of growth rings and the lumen area and cell wall and cell lumen ratio in earlywood and latewood, and the needle nitrogen concentration, were based on five seedlings per three nitrogen treatments of ambient and ozone plots ($n = 60$ or $n = 59$ due to one missing value). The relationships between the needle nitrogen content and the cambial zone and phloem widths were based on only 38 experimental seedlings, because the nitrogen content of needles was not analyzed for all those seedlings that were randomly selected for the cambial zone analysis ($n = 60$). Furthermore, due to the same reason the number of seedlings varied within the nitrogen treatments of plots ($n = 5$ to 8 seedlings per N treatment).

Results

Radial growth

Ozone and N fertilization treatments, and the year of ring formation had significant main effects on the radial stem growth of seedlings after two-year ozone exposure (Fig. 1a, Tables 2, 3, 4 and 5). The mean ± SD radius from pith to the outer boundary of 1996 ring in all studied seedlings ($n = 84$) was 2.04 ± 0.40 mm (range 1.27–3.25 mm). The width of the 1997 ring in all the N treatments was greater in the O_3-exposed seedlings as compared with that in the ambient group, and the mean width of both 1997 and 1998 rings increased in response to the HN treatment (Fig. 1a). There was also a significant interaction between N treatment and the year of ring formation (Table 5). As compared with the width of the 1997 ring, the mean 1998 ring width was smaller by about 40% in the LN treatment, and two times greater in the HN treatment (Fig. 1a). The 1998 ring width correlated positively with the N concentration of the current and previous year's needles as well (Table 6). The N concentration in the 1998 needles, ranging from 15 to 20 mg g^{-1}, was related to the increased radial width of the stem in both the control and O_3-exposed trees (Fig. 2).

Cambium and phloem

The cambial zone in the stem of Norway spruce seedlings had radially flattened cells with thin primary-cell walls which could be clearly distinguished from the secondary-phloem and secondary-xylem cells (Figs. 3a–c). The HN treatment significantly increased the number of fusiform cells and the width of the phloem and cambial zones (Fig. 3c and Tables 2 and 3). The cambial width and number of cambial cells had also correlated positively with the needle N concentrations (Table 6). Elevated O_3 did not cause any statistically significant changes in the width of cambial zone and phloem, or the number of cells in the cambium (Tables 2 and 3).

Latewood percentage

Elevated O_3 concentration significantly increased the latewood width and its percentage in the 1997 and 1998 growth rings in all the N treatments (Fig. 1c and d, Table 4). While, the high N supply significantly increased the earlywood width (Fig. 1b and Table 4). There was also significant negative correlation between latewood width and the needle N concentration (Table 6).

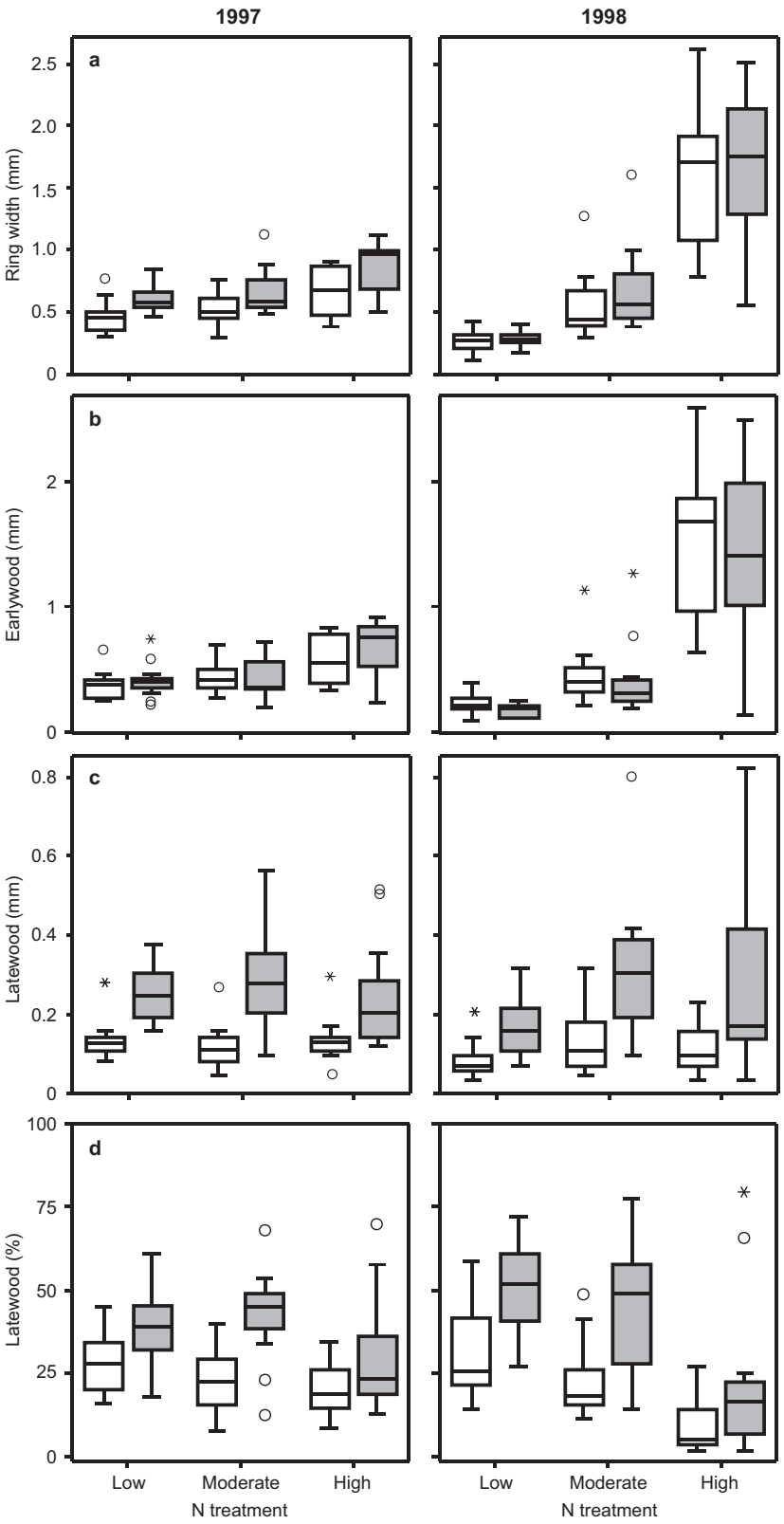

Fig. 1. Box-and-whiskers plots showing (**a**) the ring, (**b**) earlywood and (**c**) latewood widths; and (**d**) latewood percentage of ring area of 1997 and 1998 growth rings in Norway spruce seedlings after two-year ozone exposure (empty box, ambient air; grey box, elevated O_3) and N fertilization (n = 14). The line inside the box is median, its bottom and top are 25% and 75% percentiles, respectively; and the whiskers are minimum and maximum values. Asterisks and circles indicate extremes and outliers, respectively.

In all N treatments, latewood comprised approximately 20%–30% of 1997 ring area in the spruce seedlings grown under ambient O_3 (Fig 1d). The latewood percentage in 1998 ring decreased to 10% in the HN treatment but remained similar in lower N treatments. The mean latewood percentages in 1997 and 1998 rings were approximately 40%–50% in spruce grown under elevated O_3 in LN treatments, and 20%–30% in the HN treatments, respectively (Fig. 1d).

Table 2. ANOVA results for the effect of O_3 and nitrogen availability (N) on the radius of stem (mm), the cumulative width of rings (mm), the width of phloem and cambium zone, and the number of fusiform cells of Norway spruce seedlings. *F* values set in boldface indicate statistically significant effects.

Source of variation	df	Mean squares	*F*	*p*
Radius (mm)				
O_3	1	0.08	5.16	0.064
N	2	2.77	**176.41**	< 0.001
$O_3 \times N$	2	0.01	0.73	0.522
Error	6	0.02		
Cumulative width of rings (mm)				
O_3	1	0.15	**10.14**	0.019
N	2	2.80	**186.02**	< 0.001
$O_3 \times N$	2	0.17	0.01	0.850
Error	6	0.02		
Phloem (μm)				
O_3	1	0.00	0.07	0.800
N	2	0.06	**7.64**	0.022
$O_3 \times N$	2	0.01	1.26	0.349
Error	6	0.01		
Cambial zone (μm)				
O_3	1	1.75	0.06	0.851
N	2	455.43	**15.50**	0.004
$O_3 \times N$	2	59.67	2.03	0.212
Error	6	0		
Number of fusiform cells				
O_3	1	0.16	0.186	0.679
N	2	13.42	**16.26**	0.004
$O_3 \times N$	2	1.82	2.20	0.192
Error	6	0		

Table 3. The mean (± SD) radius of stem, the cumulative width of rings, the width of phloem and cambial zone, and the number of cambial fusiform cells in Norway spruce seedlings after two years exposure to slightly elevated ozone under low (LN), moderate (MN) and high (HN) nitrogen.

Treatment	Radius (mm) (n = 14)	Cumulative width of rings (mm) (n = 14)	Phloem (μm) (n = 10)	Cambial zone (μm) (n = 10)	Number of fusiform cells (n = 10)
LN					
Ambient	2.83 ± 0.35	0.81 ± 0.19	850.6 ± 142.6	22.5 ± 4.8	5.2 ± 1.2
O_3	2.88 ± 0.38	0.98 ± 0.11	980.2 ± 145.5	29.7 ± 13.0	6.4 ± 2.2
MN					
Ambient	3.32 ± 0.37	1.14 ± 0.26	1059.3 ± 231.2	27.2 ± 5.2	6.1 ± 1.1
O_3	3.57 ± 0.41	1.42 ± 0.32	998.1 ± 204.2	25.8 ± 8.7	5.6 ± 1.5
HN					
Ambient	4.40 ± 0.49	2.38 ± 0.54	1179.0 ± 141.8	48.9 ± 10.7	9.7 ± 1.6
O_3	4.60 ± 0.51	2.61 ± 0.59	1152.1 ± 163.6	40.7 ± 12.5	8.3 ± 2.4

Lumen area, cell wall/cell lumen ratio and tracheid length

The lumen area and the cell wall/lumen ratio within the earlywood and latewood were not significantly affected by O_3 (Fig. 4a–d and Table 7). There was an interaction between the year of ring formation and the N treatment in relation to the tracheid lumen area and the cell wall/lumen ratio of earlywood (Table 8). High N supply increased the lumen area of both earlywood and latewood tracheids in particular in 1998 ring (Fig. 4a–b

Table 4. ANOVA results for the effect of O_3 and nitrogen availability (N) on the ring widths (mm), and the proportion of latewood (%) in 1997 and 1998 growth rings of Norway spruce seedlings. *F* values set in boldface indicate statistically significant effects.

Source of variation	df	Mean squares	*F*	*p*
Ring width (mm)				
1997				
O_3	1	0.08	**18.71**	0.005
N	2	0.06	**13.56**	0.006
$O_3 \times N$	2	0	0.22	0.810
Error	6	0		
1998				
O_3	1	0.01	0.57	0.478
N	2	2.05	**98.12**	< 0.001
$O_3 \times N$	2	0	0.13	0.880
Error	6	0.02		
Earlywood width (mm)				
1997				
O_3	1	0	0.82	0.401
N	2	0.06	**16.03**	0.004
$O_3 \times N$	2	0	0.71	0.528
Error	6	0.004		
1998				
O_3	1	0.02	0.76	0.417
N	2	1.92	**69.84**	< 0.001
$O_3 \times N$	2	0	0.10	0.906
Error	6	0.028		
Latewood width (mm)				
1997				
O_3	1	0.05	**164.99**	< 0.001
N	2	0	0.84	0.479
$O_3 \times N$	2	0	3.53	0.097
Error	6	0		
1998				
O_3	1	0.07	**19.62**	0.004
N	2	0.01	2.71	0.145
$O_3 \times N$	2	0	1.12	0.387
Error	6	0		
Latewood percentage of ring area				
1997				
O_3	1	0.05	**32.61**	0.001
N	2	0.01	**6.70**	0.030
$O_3 \times N$	2	0	1.89	0.231
Error	6	0		
1998				
O_3	1	0.10	**31.62**	0.001
N	2	0.07	**21.88**	0.002
$O_3 \times N$	2	0	0.66	0.549
Error	6	0		

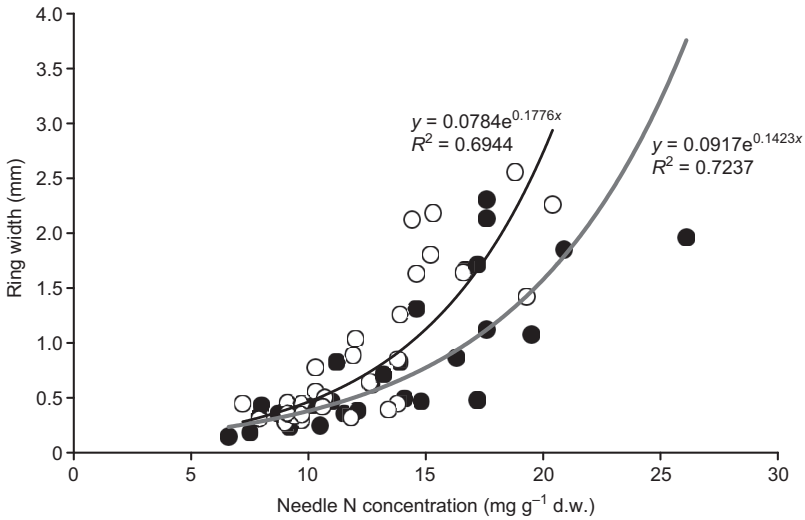

Fig. 2. The relationship between the 1998 ring width (mm) and the N concentration of current year's needles in Norway spruce seedlings after two-year open -field exposure to ambient (dots and grey line, n = 29) and elevated ozone (circles and black line, n = 31).

and Table 7). Also O_3 tended to further increase the lumen area of juvenile wood cells in the MN and HN treated seedlings (Fig. 4a–b). The lumen area (μm^2) of tracheid cells was positively correlated with the needle N concentration (Table 6). In the 1998 ring, the HN treatment reduced the

Table 5. ANOVA results for the effects of year, O_3 and nitrogen availability (N) on the ring width, earlywood and latewood widths, and the latewood percentage of Norway spruce seedlings. *F* values set in boldface indicate statistically significant effects.

Source of variation	df	Ring width (mm)		Earlywood width (mm)		Latewood width (mm)		Latewood percentage	
		F	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Year	1	**21.84**	0.001	**19.91**	0.001	0.70	0.420	0.13	0.727
Year × O_3	2	1.17	0.301	1.28	0.280	0.24	0.633	1.44	0.254
Year × N	1	**56.68**	< 0.001	**41.20**	< 0.001	2.24	0.149	**5.18**	0.024
Year × O_3 × N	2	0.19	0.829	0.32	0.735	0.71	0.512	0.21	0.811

Table 6. The correlations of two-year radial growth and wood characteristics in the 1998 growth ring, and the nitrogen (N) concentrations of the current (1998) and previous year (1997) needles (mg g^{-1} d.w.) of Norway spruce seedlings. All correlation coefficients are significant.

Stem	*n*	1998 needle N content	1997 needle N content
Radial growth (mm)	60	0.730	0.745
Ring width (mm)	60	0.775	0.786
Percentage of latewood	60	–0.354	–0.454
Cambial zone width (μm)	38	0.705	0.624
Number of fusiform initials	38	0.713	0.608
Earlywood			
Lumen area (μm^2)	59	0.407	0.509
Cell wall/cell lumen	59	–0.258	–0.329
Latewood			
Lumen area (μm^2)	59	0.488	0.462
Cell wall/cell lumen	59	–0.378	–0.366

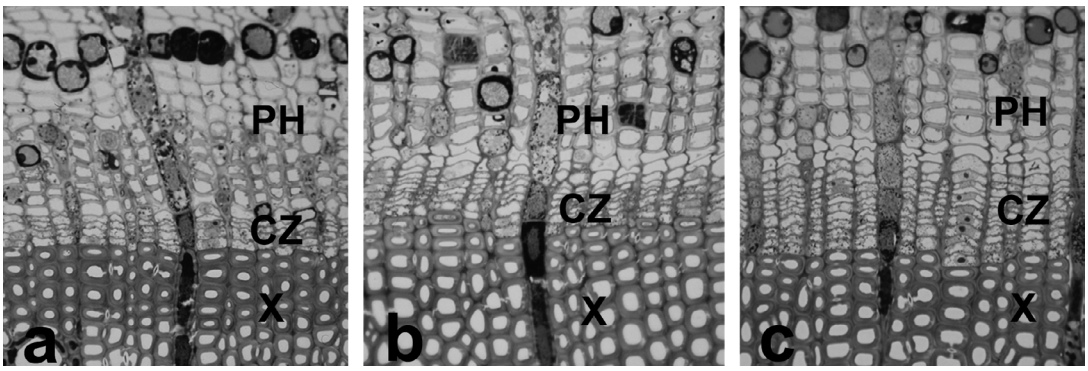

Fig. 3. Semithin cross-section through cambial zone and derivatives. Samples are from the fifth annual growth of main stem in Norway spruce seedlings after two-year (**a**) LN, (**b**) MN and (**c**) HN treatments. Cambial zone (CZ), phloem (PH), xylem (X).

cell wall/lumen ratio in both earlywood and latewood (Fig. 4c–d, Tables 7 and 8). The cell wall/lumen ratio correlated weakly and negatively with the needle N content (Table 6).

The mean tracheid length of the 1998 growth ring was not affected by O_3 or the N treatment (data not shown). The length of cells ranged from 510 μm to 570 μm in earlywood, and from 650 μm to 730 μm in latewood.

Discussion

We studied the effects of ambient and 1.5 to 1.6 × ambient O_3 concentrations on radial ring width and wood structure of stems of Norway spruce seedlings grown under different N regime during two years (1997 and 1998). The radial stem growth, ring width, and particularly the latewood percentage in stem evidently increased in response to elevated O_3 in all N treatments. Nitrogen availability regulated the earlywood formation and reduced cell wall/cell lumen ratio in both earlywood and latewood. These results confirmed the hypothesis that elevated O_3 and N alter juvenile wood production and wood structure in stems of spruce seedlings.

Radial growth

The radial stem growth in young Norway spruce seedlings was affected primarily by soil N availability. Previous results from the same seedlings (Utriainen and Holopainen 2001a) showed that nitrogen fertilization increased the current-year shoot length, total plant height, stem base diameter, biomass accumulation, and foliar nitrogen concentration. Similar positive growth response to N availability has been reported for Norway spruce (Thomas *et al*. 2005) and Scots pine (*Pinus sylvestris)* seedlings (Utriainen and Holopainen 2001a). Nitrogen amendment gives rise to fast-growing trees because of the increased vitality of their foliage (Zobel and van Buijtenen 1989). In our study, the radial stem growth was promoted at the needle N concentration range of 15 to 20 mg g^{-1} (*see* Fig. 2) which is regarded as optimal concentration for growth in most coniferous species (De Vries and Latour 1995). The significant interaction between year and N treatments revealed that N deficiency further reduced and high N supply increased the radial width of the seedlings over time.

High N availability increased xylem and phloem width in the stem of spruce seedlings suggesting stimulation of cambial zone. This is consistent with the results of Matyssek *et al*. (2002) who found similar growth stimulation in the stem tissue of well-fertilized *Betula pendula*. In our study, also the number of radial fusiform cells and consequently the cambial zone width were greater in the HN than in the LN seedlings. According to Rossi *et al*. (2012), higher accumulations of derivative cells in cambium need more time to undergo differentiation, delaying termination of cell enlargement and wall thickening and extending the length of xylogenesis.

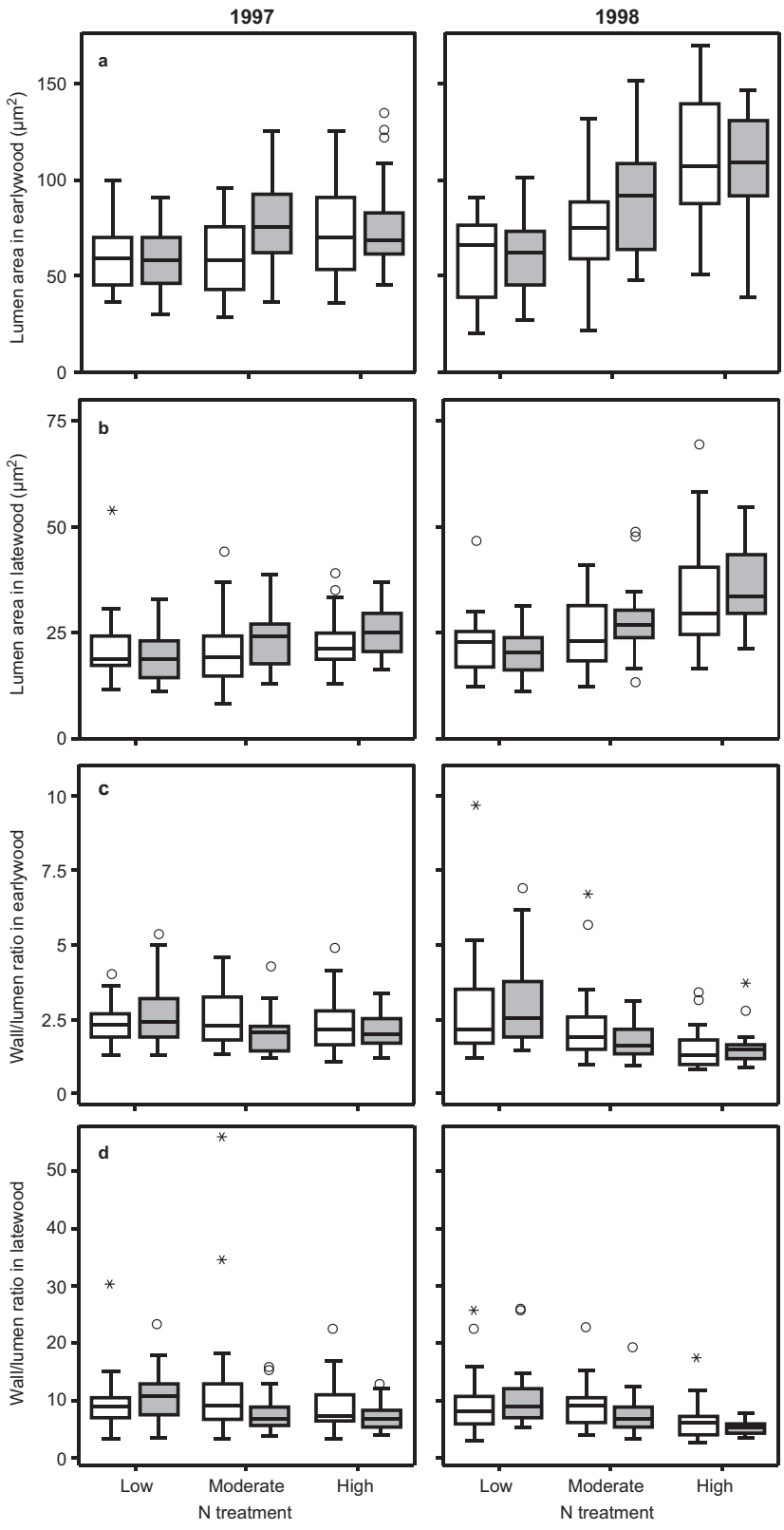

Fig. 4. Box-and-whiskers plots showing (**a** and **b**) the lumen area and (**c** and **d**) the cell wall/cell lumen ratio of earlywood and latewood tracheids within 1997 and 1998 growth rings in Norway spruce seedlings after two-year ozone exposure (empty box, ambient air; grey box, elevated O_3) under different soil N availability (n = 14). The lines in the box indicate the 25% percentile, median, and 75% percentile of the data values. The line inside the box is median, its bottom and top are 25% and 75% percentiles, respectively; and the whiskers are minimum and maximum values. Asterisks and circles indicate extremes and outliers, respectively.

Kurczyńska *et al*. (1998) found that cambial dormancy of Norway spruce seedlings occurred earlier in plants treated with low soil nitrogen than those grown under high soil N content. This suggests that HN prolonged xylogenesis in the spruce stems showing still undifferentiated cells within the cambium zone (Rossi *et al*. 2012), while in the LN seedlings cell-wall formation and maturation were mostly completed at the end of the growing season.

In addition to N enrichment, the elevated O_3 concentration increased the radial stem growth, in particular the width of ring formed during the first year of O_3 exposure. This is consistent with the findings of Utriainen and Holopainen (2001b), who reported a positive effect

Table 7. ANOVA results for the effects of O_3 and nitrogen availability (N) on the lumen area (μm^2) and cell wall/cell lumen ratio in earlywood (EW) and latewood (LW) of the 1997 and 1998 growth rings. The *F* values in boldface are statistically significant.

Source of variation	df	Mean squares	*F*	*p*
Lumen area (m^2)				
EW 1997				
O_3	1	162.14	3.25	0.121
N	2	213.73	4.29	0.070
$O_3 \times N$	2	94.02	1.89	0.232
Error	6	49.87		
EW 1998				
O_3	1	36.60	0.38	0.558
N	2	2378.5	**24.98**	0.001
$O_3 \times N$	2	62.95	0.66	0.550
Error	6	95.21		
LW 1997				
O_3	1	3.45	0.77	0.413
N	2	12.55	2.81	0.138
$O_3 \times N$	2	7.75	1.74	0.254
Error	6	4.465		
LW 1998				
O_3	1	3.99	0.36	0.573
N	2	176.57	**15.77**	0.004
$O_3 \times N$	2	3.99	0.36	0.714
Error	6	11.20		
Cell wall/cell lumen ratio				
EW 1997				
O_3	1	0.01	0.22	0.656
N	2	0.13	2.13	0.200
$O_3 \times N$	2	0.14	2.27	0.185
Error	6	0.06		
EW 1998				
O_3	1	0.05	0.18	0.687
N	2	2.01	**7.96**	0.021
$O_3 \times N$	2	0.08	0.32	0.737
Error	6	0.25		
LW 1997				
O_3	1	8.88	2.19	0.190
N	2	6.48	1.60	0.278
$O_3 \times N$	2	8.41	2.07	0.207
Error	6	4.06		
LW 1998				
O_3	1	1.51	0.75	0.420
N	2	19.48	**9.68**	0.013
$O_3 \times N$	2	0.80	0.40	0.687
Error	6	2.01		

of elevated O_3 on the stem base diameter and stem log-size (i.e. log[(stem diameter)^2 × total height]) in the same seedlings as used by us. Wellburn and Wellburn (1994) observed similar radial growth stimulation in *Pinus halepensis* exposed to 120 nl l^{-1} O_3. They found taller plants with greater stem diameters after two O_3 exposure seasons. There are also findings that slightly elevated ambient O_3 concentrations can result in a decrease (Thomas *et al*. 2005) in shoot elongation and biomass accumulation as well as have no effect on those parameters (Karlsson *et al*. 1997, Ritter *et al*. 2015) in Norway spruce seedlings. The variation in growth response to ozone among tree species and between individuals within each species can be partly explained by the different sensitivity of individuals with different genetic origin (Karlsson *et al*. 1997). Karlsson *et al*. (1997) found ozone to reduce the rate of biomass increase in faster-growing Norway spruce clone and stimulate growth in the seedlings of slower-growing clone.

Latewood formation

In the present study, wider annual ring in the O_3-exposed seedlings mainly resulted from the increased latewood production, expressed as increased latewood width and latewood percentage within the two latest growth rings. These findings suggest differences in the latewood formation process between O_3-exposed and control plants. The transition from earlywood to latewood formation occurs in late summer or autumn when cambial cell division activity and expansion declines (Uggla *et al*. 2001). The initiation of latewood formation is supposed to be triggered by shortening of the photoperiod and/or reduced temperature, and is associated with cessation of apical and needle growth at a time when current year needles have become net exporters of photosynthetic assimilate and hormones (Zobel and van Buijtenen 1989, Uggla *et al*. 2001). In boreal conifers, the carbohydrate production of new needles is high enough to meet the demand of other plant organs when the needles have reached half of their final length (Iivonen *et al*. 2001). Rossi *et al*. (2009) found that latewood initiation of mature Norway spruce occurred once shoots and needle lengthening was completed at the end of July or the beginning of August and the cell wall thickening of latewood cells was evident in mid-August. As the process of secondary wall formation in latewood cells can occur only after growth of shoots is completed (Rossi *et al*. 2009), high latewood percentage in studied O_3 and LN treated seedlings suggest early transition from earlywood to latewood followed by a longer period of latewood production.

Earlywood is formed early in the growing season (from May to July) during the active growth of trees (Uggla *et al*. 2001). In general, this is also the period with high ambient O_3 concentrations in the European boreal region (Laurila *et al*. 2004). Regardless of the potentially high level of oxidative stress during the period of earlywood formation, elevated O_3 in the present study had no main effect on earlywood growth within the growth rings. Jäggi *et al*. (2002) demonstrated with mature Norway spruce trees, that

Table 8. ANOVA results for the effect of year, O_3 and nitrogen availability (N) on the lumen area and cell wall/cell lumen ratio in the earlywood and latewood of Norway spruce seedlings. The *F* values in boldface are statistically significant.

Source of variation	df	Lumen area				Cell wall/Cell lumen ratio			
		Earlywood		Latewood		Earlywood		Latewood	
		F	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Year	1	**41.13**	< 0.001	**32.23**	< 0.001	2.53	0.113	**5.53**	0.019
Year × O_3	2	0.40	0.529	0.01	0.922	0.03	0.870	1.01	0.315
Year × N	1	**15.18**	< 0.001	**9.65**	< 0.001	**7.69**	0.001	1.40	0.248
Year × O_3 × N	2	0.07	0.930	0.04	0.960	0.20	0.820	0.71	0.493

stable carbon isotopes ($\delta^{13}C$) in earlywood of current growth year had a stronger positive correlation with $\delta^{13}C$ in starch and bulk tissue from previous year's needles than with those of the current year. This suggests that the earlywood production in the spruce seedlings was primarily regulated by N and carbohydrate status of the previous year's and older needles, and less responsive to potential disturbances in the photosynthetic assimilation of current year's needles.

In the present study, the daylight AOT40-based critical level of 5 ppm h for forest trees (UNECE 2004, van Goethem *et al*. 2013) was exceeded in the O_3 treatment during the first growing season but remained under 3 ppm h during the second growing season. The two-year exposure to elevated O_3 revealed no effects on the shoot elongation, height growth or biomass production in Norway spruce seedling as reported earlier by Utriainen and Holopainen (2001b). This suggests that the total height growth or biomass of the seedlings did not explain the O_3-induced increase in the latewood percentage of main stem. Nevertheless, the fully grown current year needles in the O_3-exposed seedlings grown under moderate or high N had a higher stomatal conductance (mean values ranged from 0.66 to 1.00 cm s^{-1}) in the late summer (measured 30 July and 31 Aug.) than those grown under ambient O_3 (mean values ranged from 0.36 to 0.48 cm s^{-1}), suggesting enhanced gas exchange (Utriainen and Holopainen 2001b) and possible photosynthesis stimulation in the new needles (Wallin *et al*. 1990). Also LN seedlings showed increased stomatal conductance (mean values ranged from 0.77 to 0.99 cm s^{-1}) in both the ambient and O_3 exposed seedlings (Utriainen and Holopainen 2001b). In all these treatments, the latewood percentage and width evidently increased (*see* Fig. 1d), which suggest that an increase in latewood formation was related to the stomatal control of gas exchange under O_3 and nutrient stress. Ritter *et al*. (2015) found that two times ambient O_3 stimulated allocation of photosynthates to stem CO_2 efflux in Norway spruce seedlings at the end of the growing season, and spruce primarily consumed newly-produced photoassimilates to stem respiration. The increase in carbon allocation to the stem was reflected by lower half-times and small sites of the carbon store (Ritter *et al*. 2015). According to Grulke (2003) at least pole-sized or larger trees can mitigate O_3-induced reductions in carbon acquisition in the summer with carbon assimilation of favourable days in the late growing season.

In the studied seedlings, tracheid length of earlywood and latewood showed no response to N supply or elevated O_3. Whereas high N supply increased the lumen area and reduced the cell wall/lumen ratio of earlywood and latewood tracheids after the second exposure season. Kurczyńska *et al*. (1998) found that during one exposure season O_3 increased the diameter of latewood tracheids in Norway spruce seedlings grown in nitrogen-enriched soils. In our study, elevated O_3 had no significant main effects on the lumen area or cell wall/lumen ratio within latewood or earlywood but it tended to increase the lumen area of latewood tracheids in the MN and HN seedlings which is in accordance with the findings of Kurczyńska *et al*. (1998). High N content in the needles likely improved the production of latewood cells by increasing the cambial activity (Zobel and van Buijtenen 1989). Due to higher xylem production the maturation of xylem cell could be delayed (Rossi *et al*. 2012) and elevated O_3 further interfered with the process of secondary wall formation resulting in tracheid cells with thinner cell walls and larger lumen area.

Environmental factors (e.g. moisture, nutrients, and climate) that change growth pattern of a tree can affect its wood properties especially wood density, mechanistic strength, and shrinkage (Zobel and van Buijtenen 1989, Rossi *et al*. 2012). The O_3-induced increase in latewood percentage was more evident in the LN treated trees, whereas the high N supply clearly suppressed the O_3 response (Fig. 1d). This suggests that the effects of O_3 and LN on the latewood formation were additive. Overall, the LN treatment resulted in smaller seedlings and slightly reduced N content in 1997 needles (Utriainen and Holopainen 2001b) resulting in slow growth of the seedlings. Wood density of spruce is related to the duration of latewood formation rather than to the length of the growing season (Zobel and van Buijtenen 1989, Uggla *et al*. 2001).This suggests that the depletion of reserves in previous-year needles

has occurred faster in the LN seedlings than in the HN plants, consequently leading to a longer period of latewood formation. The latewood width within growth rings showed little response to the additional N but the latewood percentage decreased due to the increased earlywood production. This is in agreement with the earlier observations that high soil N availability usually increases the relative amount of earlywood and has a minor effect on the amount of latewood in Norway spruce (Zobel and van Buijtenen 1989, Kostiainen *et al*. 2004).

Conclusions

This study demonstrated that environmental variables can alter the radial growth and xylem structure in the stem of Norway spruce seedlings. Elevated O_3 concentration independently of the nitrogen fertilization increased the latewood percentage in the seedlings grown under boreal climate conditions. The increase in the proportion of latewood was more obvious when the seedlings were exposed to combined O_3 and low N treatments while higher soil N supply tended to suppress the effects of O_3 on the latewood percentage. Based on the results, we concluded that exposure to slightly elevated ambient O_3 disturbs xylem differentiation in juvenile wood which may affect the wood density in the Norway spruce seedlings.

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